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THE COMPARATIVE CYTOLOGICAL EFFECTS OF SEVERAL ALKYLATING AGENTS ON HUMAN NORMAL AND NEOPLASTIC CELLS IN TISSUE CULTURE

By

JEWEL PLUMMER COBB

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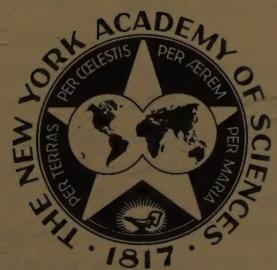

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THE COMPARATIVE CYTOLOGICAL EFFECTS OF SEVERAL ALKYLATING AGENTS ON HUMAN NORMAL AND NEOPLASTIC CELLS IN TISSUE CULTURE*

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A comparative cytological study has been made of changes produced in isolated human neoplastic and normal cells grown in tissue culture following direct exposure to one of several alkylating agents. These were: Mustargen†† (HN2), a nitrogen analogue of sulfur mustard; triethylene melamine (TEM), a polyfunctional ethylenimine triazine compound; triethylenethiophosphoramide (Thio-TEPA), a polyfunctional sulfur derivative of an ethylenimine phosphoramide compound; Chlorambucil (CB 1348), an aromatic plus butyric acid derivative of nitrogen mustard; and PAM, or phenylalanine nitrogen mustard (CB 3025), the *L*-isomeric form of an aromatic plus alanine derivative of nitrogen mustard. All five compounds have in common one or more alkylating groups capable of reacting with nucleophilic groups in the cell. The possible mechanisms involved in this reaction have been ably discussed by Price,[1] Ross,[2] Stacey *et al.*,[3] and Montgomery.[4] These compounds are frequently referred to as radiomimetic agents because of their capacity to produce certain cytological changes similar to those induced by radiation. These changes characteristically include injuries to the chromosomal mechanism frequently resulting in a typical cytomorphic pattern. Although certain similarities between the effects of these agents and those of X rays do exist,[5,6,7] there are some qualitative and quantitative differences between them.[5,6,8,9] Hence the term radiomimetic usually appears in quotes in the literature.

The alkylating capacity and subsequent cytological action observed in a variety of animal and plant systems has led to extensive use of alkylating agents at the clinical level. Clinical experiences with HN2, TEM, Thio-TEPA, and CB 1348 have been ably summarized by a number of clinical investigators[10,11,12,13] in the monograph entitled *Comparative Clinical and Biological Effects of Alkylating Agents.*[14] Creech *et al.*[15,16] have had moderate success with the use of PAM in chemotherapy of advanced neoplasms via isolation-perfusion techniques.

*The investigation reported in this paper was supported in part by research grants from the National Cancer Institute, Public Health Service, Bethesda, Md., and from the Damon Runyon Memorial Fund for Cancer Research, Inc., New York, N.Y.

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††The trade name of Merck, Sharp and Dohme, Philadelphia, Pa., for nitrogen mustard is Mustargen.

The studies described in this report were designed to reveal morphological sites of drug action, bearing in mind possible similarities and differences among the compounds. Initial experiments were conducted to evaluate and compare cellular changes produced in HeLa cells following exposure to each agent for 1 to 24 hours. These data established threshold dose values that were used for studies on primary short-term tissue cultures of human neoplasms. Comparisons were made among the five compounds with reference to their ability to produce cytostatic, mitoclastic and/or cytocidal changes in HeLa cells and in a variety of primary short-term human neoplastic tissue cultures.

Materials and Methods

Stock cultures of HeLa cells* were serially propagated in Kimax milk dilution bottles by scraping the cells off the glass, breaking up the cell clumps with a fine bore pipette and transferring the suspended cells at a dilution of 100,000 cells/ml. Cell counts were determined with a Neubauer bright-line hemacytometer. The transfer solution consisted of 50 per cent conditioned medium and 50 per cent fresh medium. The medium, composed of 90 per cent Eagle's medium (EBM) and 10 per cent pooled, undialyzed, and uninactivated human serum was always adjusted to *p*H 7.2 before use. When cells were to be used for drug studies they were dispersed into Porter flasks containing cover slips. The medium with cells was apportioned in 1-ml. aliquots. Flasks with cells were incubated at 37° C. for two to three days prior to use in drug studies.

Human neoplastic and normal tissue was excised by sterile procedures from patients with disseminated or advanced neoplastic disease. Such material was cultured, using standard techniques described in a previous report.[17] In this group only actively growing primary cultures, growing from 1 to 36 days *in vitro*, were employed for both control and drug-study series. Only cultures with a minimum cell population of 1,000 cells per sample were selected for drug experiments.

The chemotherapeutic agents employed in this tissue culture investigation were prepared in the following manner. One mg. per ml. of sterile, crystalline HN2·HCl was dissolved in distilled water and stored at −10° C.; aliquots were thawed just before use. The TEM and Thio-TEPA† were obtained in crystalline form, weighed, dissolved in distilled water, sterilized by filtration through a Corning fritted-glass

* Obtained from the Carver Research Foundation, Tuskegee, Ala. and from The John L. Smith Memorial for Cancer Research, Maywood, N.J., and from John Davenport and Irving Toplin of Chas. Pfizer & Co., Inc., Maywood, N.J.

† Furnished through the courtesy of Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N.Y.

filter of UV porosity, and stored at −10° C. The CB 1348,* obtained as a sterile crystalline powder, was dissolved in a small amount of 70 per cent ethanol and then in a solution of disodium phosphate, thus converting it to the soluble sodium salt. Similarly, the stock solution was stored at −10 C. The PAM* was obtained in crystalline form, dissolved in 0.1 *N* HCl and filtered as described above. The sterile acid filtrate was stored immediately at −10° C. Just before use an aliquot was thawed, and each 2 ml. of the filtrate was combined with 2.5 ml. of 0.1 *N* NaOH solution. Stock concentrations of each of these compounds were diluted several times in nutrient medium for final use in cultures as 1 to 9 dilutions.

The cytological terms used in this report are those cited by Madsen,[7] and are defined as follows: cytocidal, direct damaging effect(s) on all the cells of the culture, whether interphasic or dividing; cytostatic, prevention of cells from entering mitosis; and mitoclastic, effect(s) on cells during mitosis, for example, pycnosis, fragmentation, stickiness, clumping, and other chromosomal aberrations.

The following data were recorded from stained slide preparations for each experimental series: (1) per cent of mitoses, (2) per cent of various mitotic stages, (3) types and per cent of chromosomal aberrations, (4) per cent and size of giant cells and, (5) per cent of pycnosis and cytolysis. Threshold concentrations were expressed in terms of the least injurious dose (LID), defined as the lowest concentration causing a significant reduction in per cent of mitoses and an increase in aberrant chromosomal structures in 24 hours.

In several experiments aimed at the production of giant cells the giant cell producing dose (GCPD) was determined. This was defined as the lowest concentration which produced a population composed primarily of giant cells at 15 to 17 days following a 24 hour exposure to the drug. In order to determine the GCPD, HeLa cells were exposed for 24 hours to a range of dilutions of each drug, then washed with Hank's saline solution, re-fed with fresh media, and returned to the incubator for 17 days. During the posttreatment period they were fed three times per week. The time of 17 days was chosen to compare these results with those of Puck and Marcus,[18] who observed post-X-irradiation changes in HeLa cells. Measurements of nuclear diameters of 100 cells selected at random were taken to determine the proportion and size of giant cells in the population. Cells with nuclear diameters ranging from 10 μ to 18 μ were designated giant types. It is realized that the nuclear diameter is not always an accurate indication of the change in the entire cell area; however, the cytoplasmic processes were often stretched

* Furnished through the courtesy of Burroughs Wellcome, Tuckahoe, N.Y.

so thin as to be almost invisible. There appeared to be "good" correlation between the nuclear increase and cytoplasmic increase. Cells with enlarged nuclear diameters always displayed increased cytoplasmic areas.

HeLa cultures were exposed to drugs for varying periods of time, depending upon the design of the experiment. Primary neoplastic cultures were exposed to threshold concentrations of the drugs for 96 hours, when they were fixed and stained. Routinely, all HeLa cultures and primary tissue cultures included in the control and drug studies were fixed with methanol and stained with Jenner-Giemsa. Living cultures were examined under the ordinary light microscope or the phase-contrast microscope.

Effect of Alkylating Agents on Mitosis in HeLa Cells

Studies were undertaken to investigate the cellular changes produced in HeLa cells following exposure to drug for 24 hours. These observations were made to determine and compare the nature of cellular changes. The least injurious dose (LID) was obtained for each drug. The following cellular phenomena were recorded: (1) alterations in per cent of mitoses, (2) alterations in proportions of each mitotic stage, (3) types of physical chromosomal damage, (4) incidences of pycnotic and of cytolyzed cells, and (5) incidences of giant cells as compared to controls.

A summary of the changes in treated cells exposed to HN2, TEM, Thio-TEPA, CB 1348, and PAM, at concentrations ranging from 1 mM to 1×10^{-4} mM, is presented in TABLE 1. HN2, TEM, and PAM, at 1 mM concentrations, caused total cytolysis of HeLa cells. The same concentration of Thio-TEPA did not cause total cytolysis, but produced a sixfold reduction in mitosis. CB 1348, at the same concentration, caused an over-all clumping of nondividing cells. There was a decrease in the staining capacity of all cells left on the cover slip.

As recorded in TABLE 1, HN2 produced cytotoxic changes at concentrations ranging from 0.1 mM to 5×10^{-4} mM. A high percentage of cells in division displayed scattered or clumped chromosomal structures at the metaphase stage. There was also a marked increase in cellular debris. The LID was found to be 5×10^{-3} mM.

TEM produced cytotoxic changes at a concentration of 0.1 mM. The percentage of mitoses was reduced almost fivefold. Cells in division displayed scattered or clumped chromosomal structures which were similar to those produced by HN2. At lower concentrations, however, the damage was much less severe than that seen when HN2 was used in similar doses. The LID was 5×10^{-2} mM. In the case of TEM at 1×10^{-4} mM concentration, the per cent of mitoses exceeded that of the controls. An examination of the per cent of each mitotic stage indicated

an apparent increase in all stages rather than a piling up due to damage at one specific stage. This concentration was considered to be a true stimulatory dose.

Thio-TEPA at a concentration of 0.5 mM produced marked cytotoxic changes and caused a sixfold reduction in mitoses and an increase in cellular debris. The cells in mitosis showed aberrant metaphases primarily. Normal metaphases as described by Moorhead and Hsu[19] were not observed. Concentrations of 0.1 and 1×10^{-2} mM caused a definite reduction in mitoses and a high percentage of metaphases. At a concentration of 0.1 mM, more than 50 per cent of these metaphases were aberrant and tended to resemble those structures observed in cultures treated with HN2 and TEM. The LID was 0.1 mM. Additional extensive studies using Thio-TEPA have been presented elsewhere.[20] Briefly, a 5 mM concentration exposed to HeLa cells for 24 hours produced general cytolysis. Thio-TEPA at a 1 mM concentration caused a marked reduction in the number of mitoses. Delayed chromosomal damages, consisting of fractionation or superfragmentation of chromosomes, appeared 96 hours after drug removal. All dividing cells had chromosomal aberrations and all cells were larger than the control cells. Normal dividing cells were not seen seven days after removal of the drug. Seventeen days later there was a predominance of giant mono- and multinucleated cells similar to those formed after X irradiation. Exposure for 96 hours at lower doses resulted in a marked increase in the number of aberrant metaphases. Exposure for only 1 hour to a concentration of 1 mM was sufficient to produce typical immediate and delayed cellular changes.

CB 1348 at concentrations equivalent to those cited above produced milder cytological changes. Doses of 0.5 mM to 0.1 mM caused a reduction in mitoses. The LID was 0.5 mM. The increase in aberrant metaphase structures was not as great as that observed with LID values of HN2 and Thio-TEPA.

PAM caused over-all cytolysis and subsequent loss of cells from the cover glass at a 0.5 mM dose. A concentration of 0.1 mM produced a marked reduction in mitoses and a significant increase in aberrant metaphase structures. The LID was 0.1 mM. Lower concentrations produced an apparent increase in mitosis. A more detailed examination revealed that the increase in mitosis at 0.01 mM was due to an increase in abnormal metaphases. At 1×10^{-3} mM the number of all stages was increased. This was considered to be a true stimulatory dose. The type of chromosomal aberration resembled that found in other cultures treated with HN2, TEM, Thio-TEPA, or CB 1348. A photomicrograph of such an aberrant metaphase structure is seen in FIGURE 2, and the control is seen in FIGURE 1.

TABLE 1

MITOTIC CHANGES IN HeLa CELLS FOLLOWING 24 HOUR EXPOSURE TO ALKYLATING AGENTS*

Drug	Concentration (μg./ml.)	Concentration (mM)	Mitoses (%)	Prophases (%)	Normal metaphases (%)	Fractured metaphases (%)
HN_2HCl	Control		4.9	14.3	59.2	0
	192.5	1.0	0	SEVERE CELL INJURY		
	19.25	0.1	0	SEVERE CELL INJURY		
	9.62	0.05	1.7	0	0	11.8
	0.96	0.005†	1.3	0	30.7	7.7
	0.09	0.0005	3.3	15.00	39.0	0
TEM	Control		4.9	14.3	59.2	0
	204.2	1.0	0	CYTOLYSIS		
	20.4	0.1	1.0	10.0	10.0	0
	2.0	0.01†	2.6	0	70.0	0
	0.02	0.0001	5.3	13.2	47.16	0
	Control		5.3	11.3	52.8	0
	10.2	0.05	4.4	0	50.0	0
	0.20	0.001	4.5	6.66	57.7	0
Thio-TEPA	Control		5.3	11.3	52.8	0
	189.2	1.00	0.9	22.2	22.2	0
	94.6	0.5	0.6	0	0	66.6
	18.9	0.1†	1.2	0	41.66	0
	1.89	0.01	3.7	5.4	43.2	0
CB 1348	Control		5.3	11.3	52.8	0
	304.2	1.0	0	CYTOLYSIS		
	152.1	0.5†	3.5	8.57	31.42	0
	30.4	0.1	4.0	7.5	42.5	0
PAM	Control		6.0	10.0	58.3	0
	304.3	1.0	0	CYTOLYSIS		
	152.1	0.5	0	CYTOLYSIS		
	30.4	0.1†	1.3	30.76	15.38	0
	3.04	0.01	6.4	20.3	39.06	1.56
	0.30	0.001	7.0	8.57	54.28	12.8

* Percentages are based on counts of 1,000 cells for each sample.

† Least injurious dose.

MITOTIC CHANGES IN HeLA CELLS (*continued*)

Other abnormal metaphases (%)	*Normal anaphases (%)*	*Telophases ‡ (%)*	*Pycnotic cells (%)*	*Cytolyzed cells (%)*	*Giant cells (%)*
10.2	10.2	6.1	0.5	0.1	0
		SEVERE CELL INJURY			
		. SEVERE CELL INJURY			
88.2	0	0	73.0	0	0
61.5	0	0	0.5	0.7	0
46.0	0	0	0.8	0.7	0.1
10.2	10.2	6.1	0.5	0.1	0
		CYTOLYSIS			
70.0	0	10.0	0.3	0.2	0.2
11.0	0	19.0	0.4	0.3	0
16.98	15.1	7.54	0.1	0.4	0
3.77	11.3	20.8	0.1	0	0
34.09	0.1	6.8	0.1	0	0
15.55	13.3	6.66	0.2	0	0
3.7	11.3	20.8	0.1	0	0
44.4	0	11.11	0	0	0
16.66	0	16.66	0	0	0
50.0	8.33 §	0	0	0	0
21.6	0	5.4	0.2	0	0
3.7	11.3	20.8	0.1	0	0
		CYTOLYSIS			
14.28	5.71	11.42	0.1	0	0
20.0	7.5	20.0	0.1	0.3	0
0	2.5 §				
5.0	5.0	21.66	0.1	0	0.5
		CYTOLYSIS			
		CYTOLYSIS			
30.76	0	23.07	0.2	0	1.9
14.06	4.68	20.03	0.1	0	1.0
0	8.57	15.7	0.1	0	0.9

‡ Includes telophases and postphases as described by Moorhead and Hsu.[19]

§ Percentage of abnormal anaphases.

FIGURE 1. Control HeLa cell culture with two normal metaphase stages. ×733.

All photomicrographs illustrating this paper are of cells stained with Jenner-Giemsa.

FIGURE 2. HeLa cells 24 hours after exposure to LID of HN2. Note aberrant chromosomal structure in metaphase stage, characteristic of damage caused by all alkylating agents studied. ×733.

Comparative Study of Reversibility of Cellular Injury Following Brief Exposure to Equal Millimolar Concentrations of Alkylating Agents

HeLa cells were exposed for 1 hour to a 1 mM dose of each of the agents under study. This dose and time were chosen after analysis of earlier studies of Thio-TEPA on HeLa cells revealed that exposure to a concentration of 1 mM for one hour was sufficient to produce cellular injury. At the end of 1 hour the cultures were washed, re-fed with drug-free medium, and returned to the incubator for 96 hours. The cells were then fixed and stained.

The results of the 1-hour exposure of the alkylating agents to HeLa cells are summarized in TABLE 2. An examination of cultures 96 hours after the removal of the drugs indicated that all 5 drugs produced damage within 1 hour. Acute differences between the cytotoxic properties of equivalent millimolar concentrations of the agents were apparent. HN2 and PAM caused cytolysis of the total cell population resulting in the death and detachment of the cells from the cover slip. TEM caused severe cellular injury manifested by a partial loss of cells from the glass, pycnosis and increased cellular debris. No mitoses were observed. The cells that remained attached to the cover slip were giant cells with nuclear diameters of about 24 μ. The nuclear diameters of the control cells averaged 10 μ. Thio-TEPA, on the other hand, produced milder changes. The mitotic incidence was reduced almost 50 per cent.

TABLE 2

EFFECT ON HELA CELLS FOLLOWING EXPOSURE FOR 1 HOUR TO 1 MILLIMOLAR CONCENTRATIONS OF VARIOUS ALKYLATING AGENTS*

Drug	*Mitoses* † (%)	*Normal mitoses* (%)	*Abnormal mitoses* (%)	*Giant cells* (%)	*Pycnosis* (%)	*Cytolysis* (%)
Control	4.4	90.91	9.09	0	0.4	0.6
HN2	0			0		100
TEM	0			100		
Thio-TEPA	2.9	24.0	76.0	100	2.3	1.1
CB 1348	15.0	1.3	98.7	100	1.6	0.7
PAM	0			0		100

* Following 1-hour exposure to each drug, the cells were re-fed with fresh medium and returned to the incubator for 96 hours, at which time they were fixed and stained.

† One thousand cells counted for each sample.

Seventy-six per cent of the dividing cells (all metaphases) were abnormal, and showed scattered and fractured chromosomes. The incidence of pycnotic and cytolyzed cells was increased. The entire population, both interphasic and dividing cells, consisted of giant cell types with an average nuclear diameter of 20 μ. CB 1348 caused an increase in the number of mitoses. This was only an apparent increase, since all dividing cells were in metaphase and 98.7 per cent of these were aberrant. The chromosomal damage consisted either of a wide scattering and fractionation of the chromosomes, or a condensation of chromatin to form one or two irregular eccentric masses. Again, as with Thio-TEPA, all cells were enlarged with an average nuclear diameter of 22 μ. The number of pycnotic cells was increased. Since striking cellular changes were observed 96 hours after drug removal, the damage produced by all five agents was irreversible.

Giant Cell Studies

The formation of giant cells, observed 17 days following exposure of HeLa cells to Thio-TEPA, was described in an earlier report.[20] Giant cell formation *in vitro* was similar to that observed after irradiation by several workers. In tissue culture all five agents produced giant cells resembling those formed following X irradiation. The drug concentration necessary to produce a population composed primarily of giant cells was found to vary according to the agent used (TABLE 3). The GCPD was usually slightly higher than, or equivalent to, the LID. Giant cell nuclei ranged in size from 21 μ to 53 μ. The GCPD ranged from 1 mM for Thio-TEPA to 1×10^{-2} mM for PAM. Seventy-two to 96 per cent of the cell nuclei in drug-exposed cultures were of the giant type, whereas only 22 per cent of the cells in control cultures were of the giant type (FIGURES 3 and 4). The nuclear size of "normal" cells observed in drug cultures was 16 μ, as compared to 13 μ observed in control cultures. In cultures exposed to Thio-TEPA, those cells which did not change to giant cells were often smaller than the controls, and probably represented a stage of pycnosis.

The morphology of the giant cells produced by each drug was essentially similar. Giant cells were usually mono- or binucleate with densely staining nuclei. The nuclei frequently contained giant (7 to 11 μ) nucleoli (FIGURE 5). The cytoplasm was basophilic, pale-staining, and devoid of definitive granules, although occasional vacuoles were present. Atypical structures in giant cells consisted of intranuclear vacuoles, fragmented nuclei and multiple micronuclei (FIGURES 6, 7, 8) similar to those observed in giant cells formed after X irradiation. The enlargement process was observed to begin as early as three days after drug exposure. The main difference between a 6-day and a 17-day post-

TABLE 3

COMPARATIVE SIZES AND DISTRIBUTION OF GIANT CELL NUCLEI FORMED AFTER EXPOSURE TO ALKYLATING AGENTS*

Drug	*Dose in mM*	*Giant nuclei (%)*	*Average size of giant nuclei (range) μ*	*Normal nuclei (%)*	*Average size of normal nuclei (range) μ*
Control	-	22	23 (21 - 30)	78	13 (7 - 18)
HN2	0.5	86	25 (21 - 49)	14	16 (11 - 18)
TEM	0.5	92	32 (21 - 39)	18	14 (11 - 18)
Thio-TEPA	1.0	94	31 (21 - 49)	6	10 (10)
CB 1348	0.5	72	33 (21 - 53)	28	16 (11 - 18)
PAM	0.01	80	30 (21 - 53)	20	16 (14 - 18)

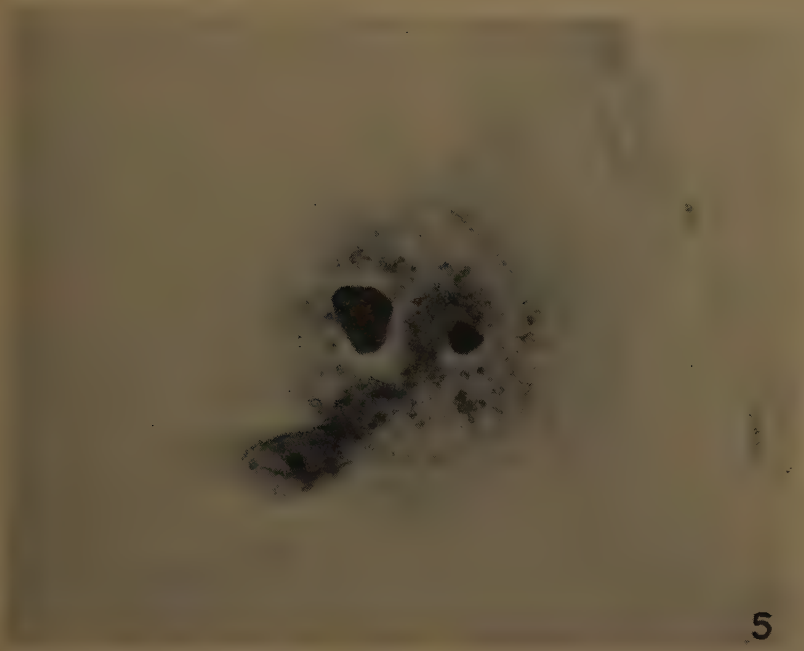
* One hundred nuclei counted per sample.

treatment culture, for example, appeared to be a decrease in the number of cells attached to the glass surface. The number of cells that remained attached to the cover slip at 17 days varied from drug to drug. All drugs tended to cause a marked shift in the distribution of cell sizes toward the larger type. This resulted in a greater range of cell sizes.

Intranuclear vacuoles were most common in Thio-TEPA, CB 1348 and HN2 exposed cultures. Multiple micronuclei and bizarre nuclear fragmentation were most common with TEM, Thio-TEPA, HN2, and PAM. Cells with aberrant mitotic structures remained attached to the cover slip only in PAM-treated cultures. The widest range of bizarre nuclear forms and multinucleated cells was found in Thio-TEPA cultures. In all drug series a reduction in concentrations by one half to one tenth of a unit produced populations with less than 50 per cent giant cells. Enlarged cells approaching giant type dimensions were observed in some of the primary tissue cultures of human normal, and neoplastic tissue were exposed for 96 hours to threshold concentrations of Thio-TEPA and CB 1348. Cell enlargement, when observed, was found only in those drug-exposed cultures that showed a large number of mitoses prior to treatment. Some of the culture series with enlarged cells included a fibrosarcoma, a Hodgkins' disease lymph node, a malignant lymphoma, and a normal lymph node (FIGURES 9 to 16). Frequently, enlarged cells showed

FIGURE 3. Control HeLa cell culture. ×195.

FIGURE 4. Giant HeLa cells, 17 days after a 24-hour exposure to 0.01 mM PAM. ×195.



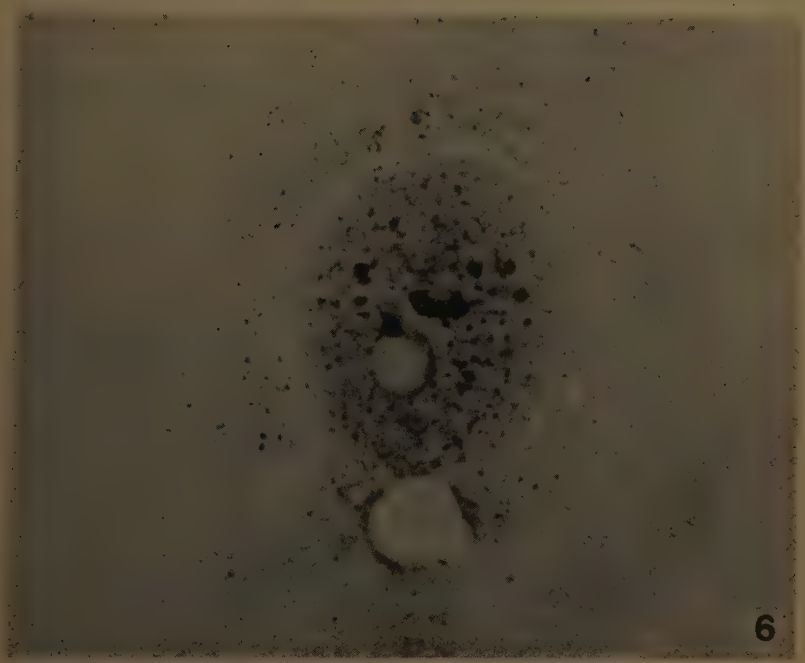


FIGURE 5. Giant HeLa cell nucleus with nucleoli, 17 days after exposure to 0.01 mM PAM. Compare size with FIGURE 1. ×740.

FIGURE 6. Intranuclear vacuole in giant HeLa cell, 17 days after exposure to 0.5 mM CB 1348. ×740.

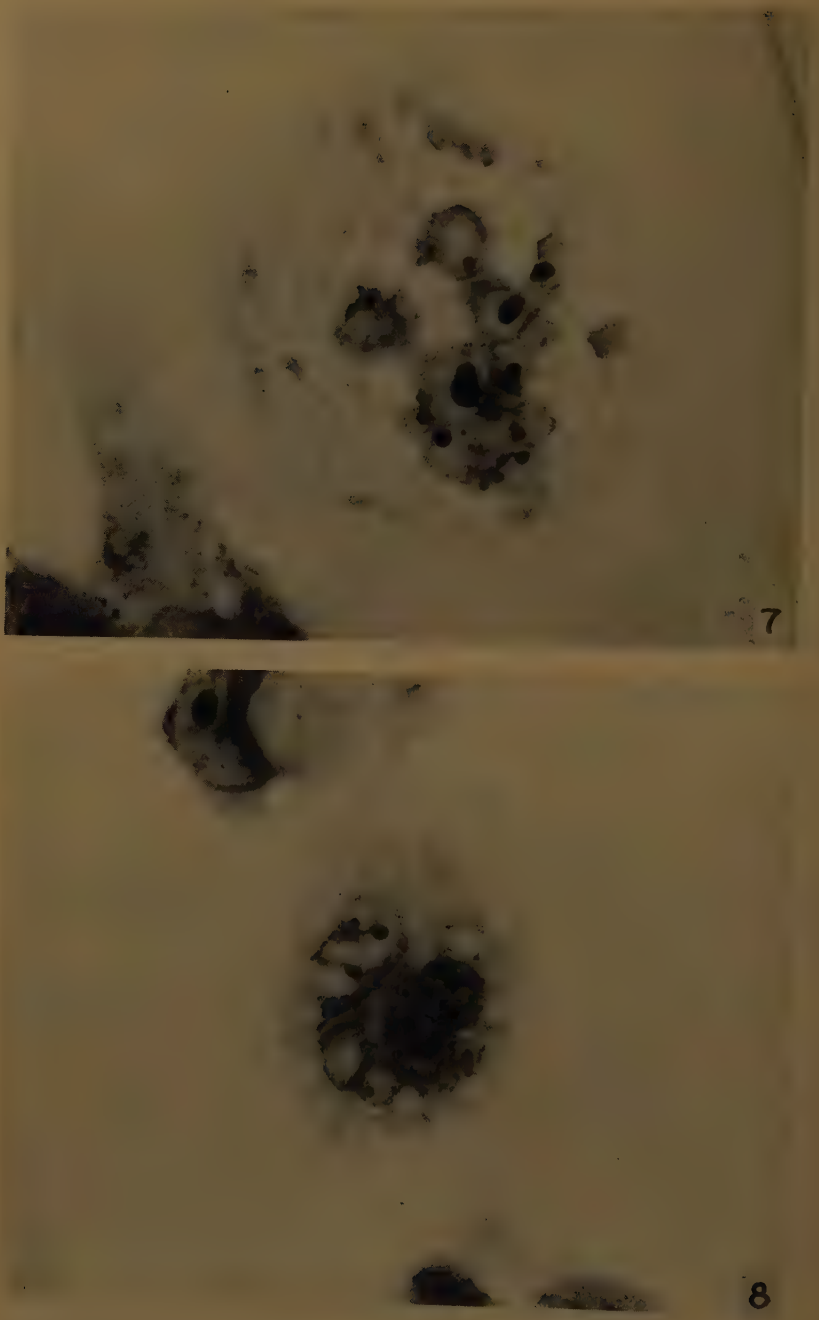


FIGURE 7. Nuclear fragmentation in giant HeLa cell, 17 days after exposure to 0.01 mM PAM. ×764.

FIGURE 8. Multiple nuclei in giant HeLa cell, 17 days after exposure to 0.01 mM PAM. ×764.

FIGURE 9. Control fibrosarcoma culture, showing tripolar mitosis and bipolar cell growth pattern. ×263.

FIGURE 10. Treated fibrosarcoma culture after 96 hour exposure to 0.5 mM CB 1348. Note cell enlargement, nuclear fragmentation, and loss of cell polarity. ×263.

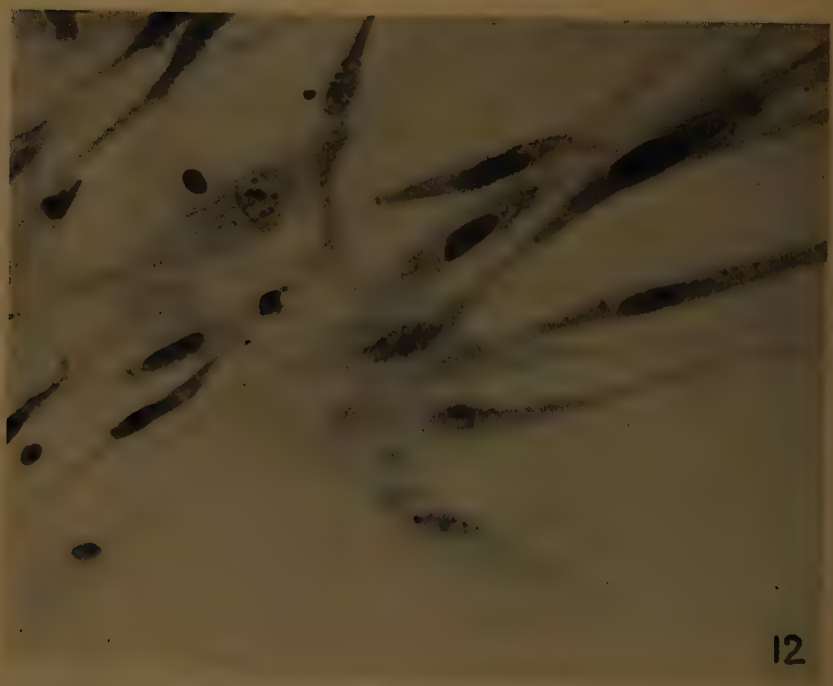


FIGURE 11. Control Hodgkins' disease lymph node culture. ×256.

FIGURE 12. Treated Hodgkins' disease lymph node culture after 96 hour exposure to 0.5 mM Thio-TEPA. Note enlarged reticular cell types. ×256.

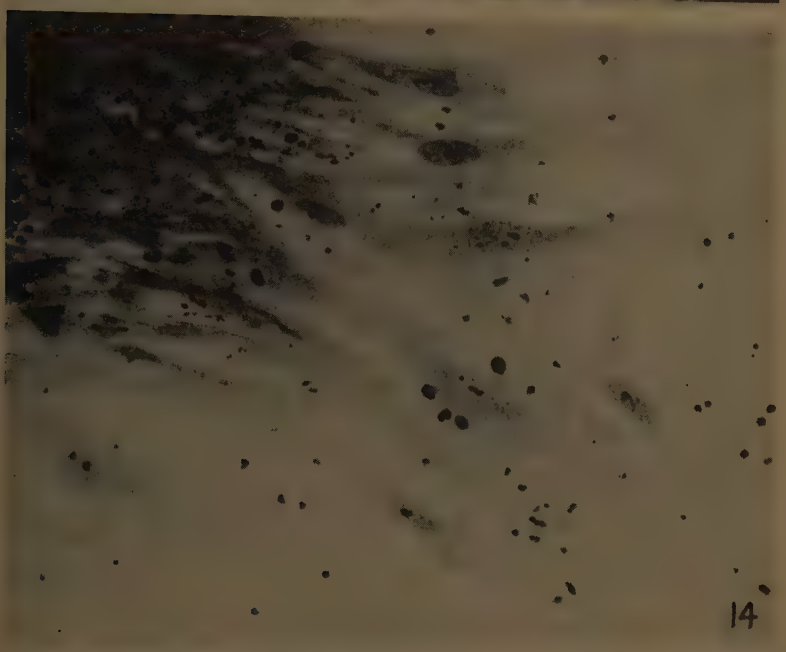


FIGURE 13. Control malignant lymphoma lymph node culture. Note 3 dividing cells in metaphase. ×225.

FIGURE 14. Treated malignant lymphoma lymph node culture after 96-hour exposure to 0.5 mM CB 1348. Note enlarged reticular cells and pycnotic lymphocytic elements. ×225.

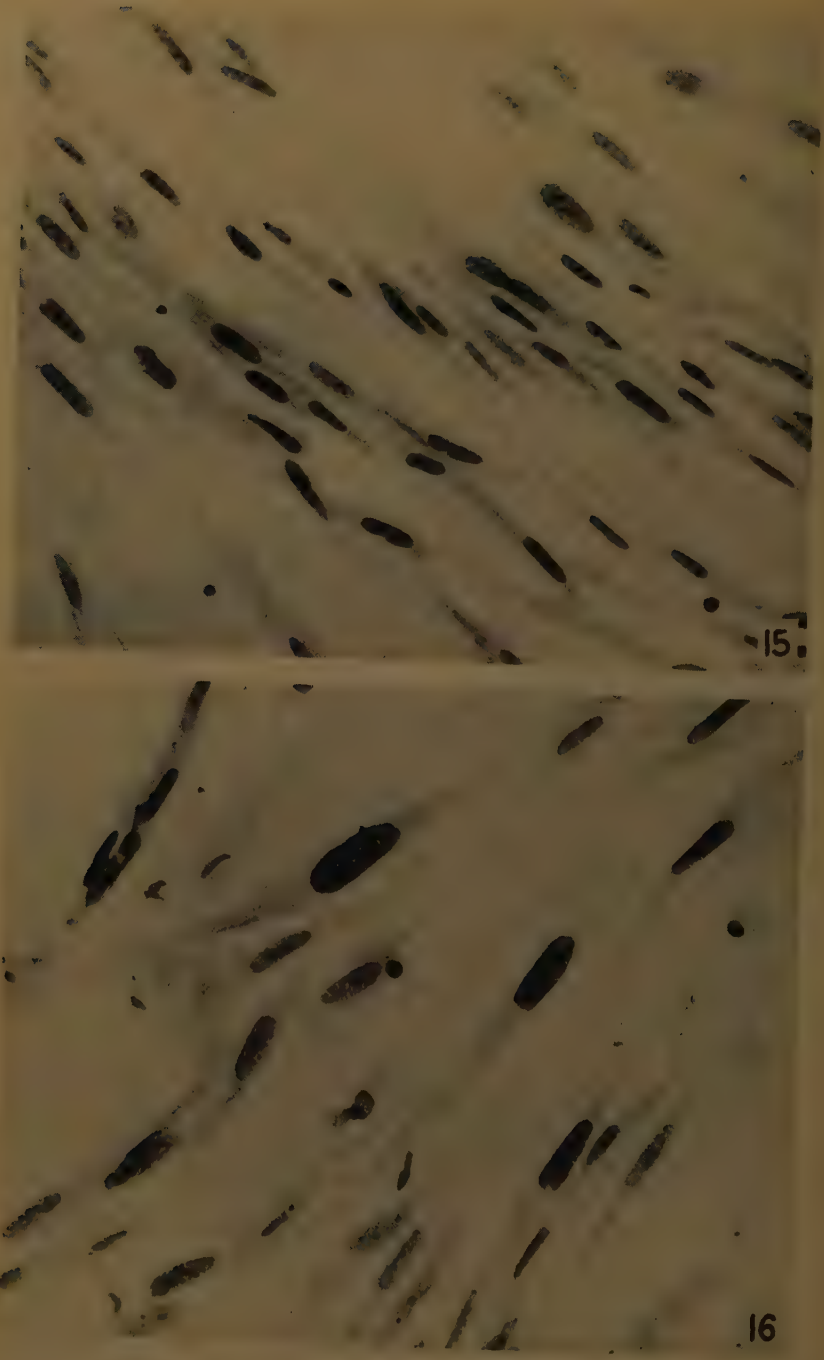


FIGURE 15. Control normal lymph node culture. ×250.

FIGURE 16. Treated normal lymph node culture after 96 hour exposure to 0.5 mM Thio-TEPA. Note cell enlargement and random pycnosis. ×250.

the multiple micronuclei and nuclear fragmentation seen in treated HeLa cultures.

Cellular Changes in Short Term Primary Cultures of Human Normal and Neoplastic Tissues Treated with Thio-TEPA, CB 1348, and PAM

Cultures growing on cover slips were exposed to 0.5 mM or 0.1 mM Thio-TEPA,* 0.5 mM CB 1348, and 0.1 mM PAM for 96 hours, when they were fixed and stained. Those cells having cellular injury at 96 were considered sensitive; cells with either questionable or no damage were evaluated as resistant. The criteria used for the evaluation of cellular damage included changes considered to be (1) cytocidal (damage to all cells of a given population), for example, granulation, vacuolation, pycnosis, loss of staining capacity, and increased cellular debris; (2) cytostatic (suppression of mitosis); and (3) mitoclastic (chromosomal aberrations).

TABLE 4 summarizes and compares the cellular changes observed in 222 primary neoplastic tissue cultures exposed to Thio-TEPA, CB 1348, or PAM. Sensitivity of cells to each drug is recorded in the *S* column; resistance or questionable damage is recorded in the *R* column. The changes observed with the three drugs were similar and comprised a range of cytological alterations, including granulation, pycnosis of all or

TABLE 4

TOTAL NUMBER OF TISSUE CULTURE CASES TREATED WITH THIO-TEPA, CB 1348 AND PAM

Type of tissue	*Thio-TEPA*		*CB 1348*		*PAM*	
	R	*S*	*R*	*S*	*R*	*S*
Lymphomas	8	13	10	20	7	3
Melanomas	11	9	2	6	4	0
Carcinomas	15	25	18	18	7	6
Sarcomas	5	8	3	4	1	2
Tumors of nervous system	1	5	2	5	2	0
Mesotheliomas	0	1	0	1	-	-
Normal	1	3	2	1	3	1
Total number neoplastic	40 (39.6%)	61 (60.4%)	35 (39.3%)	54 (60.7%)	21 (65.6%)	11 (34.4%)

*Earlier unpublished studies comparing 0.5 mM and 0.1 mM concentrations of Thio-TEPA indicated that they were approximately equal in effectiveness in the 1° culture test system employed. All those cultures sensitive to 0.5 mM were also sensitive to 0.1 mM. Similarly all those resistant to one dose were resistant to the other.

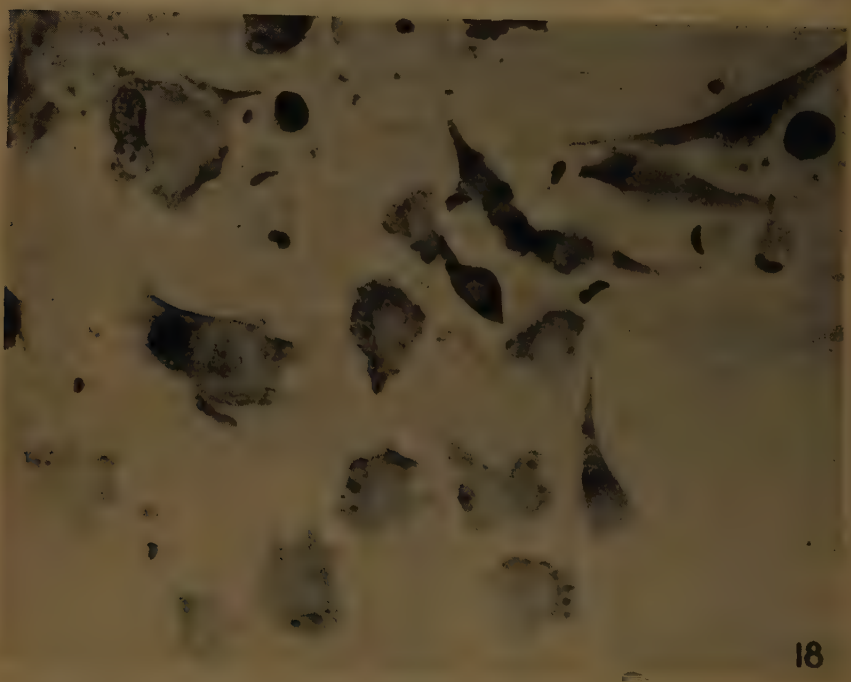


FIGURE 17. Control basal cell carcinoma culture. ×257.

FIGURE 18. Treated basal cell carcinoma culture after 96-hour exposure to 0.1 mM Thio-TEPA. Note cellular damage to carcinoma cells. ×257.

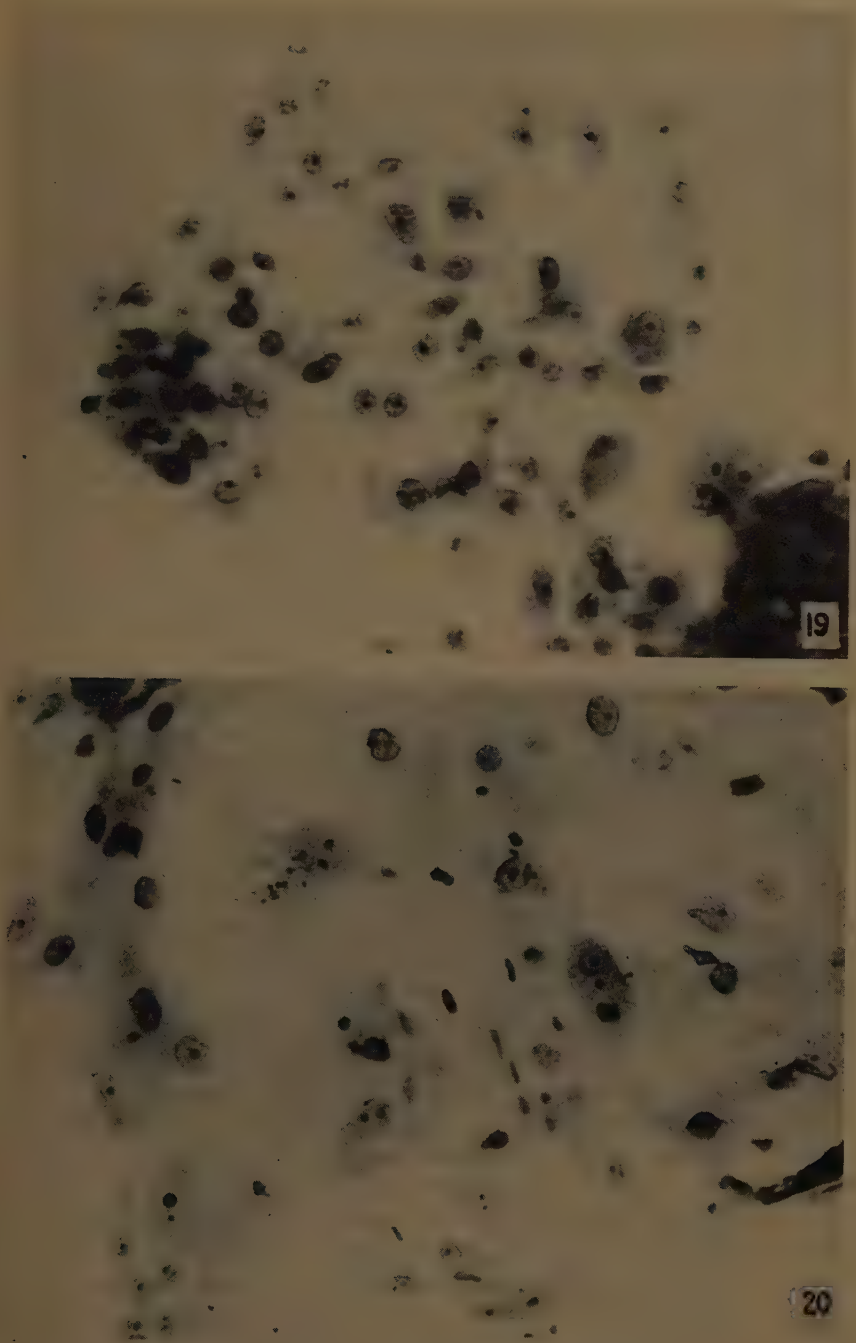


FIGURE 19. Control squamous cell carcinoma culture. ×266.

FIGURE 20. Treated squamous cell carcinoma culture after 96-hour exposure to 0.5 mM CB 1348. Note cell enlargement and random pycnosis. ×266.

a few cells of the population, complete or partial suppression of mitoses, cell enlargement, nuclear fragmentation, loss of staining capacity, and chromosomal aberrations. Each drug caused a suppression of mitoses in all cultures that displayed mitoses prior to drug exposure. Chromosomal aberrations consisting of pycnosis, breakage, and clumping were often observed. Thio-TEPA caused cellular damage in a majority of carcinoma, sarcoma, lymphoma, and neural neoplastic tissue culture series. One type of cellular injury caused by Thio-TEPA is shown in FIGURE 18 with the control cell type shown in FIGURE 17. CB 1348 caused cellular damage in a majority of the lymphoma and neural neoplastic tissue series, and in 50 per cent of the carcinoma series. An example of damage to the cells caused by CB 1348 is shown in FIGURE 20, while the control cell type is shown in FIGURE 19. PAM caused a similar type of cellular damage in relatively few of the neoplastic tissue series tested.

The degree of damage elicited by CB 1348 and PAM (columns 2 and 3) on neoplastic cells was compared. CB 1348 caused more damage when the results were compared with the percentage of cases sensitive to PAM. This difference was proved to be statistically significant at the 1 per cent level, using the Chi-square test of significance. The per cent sensitivity values for PAM and Thio-TEPA were compared. Thio-TEPA was found to be the most effective agent at the 1 per cent level. When the results with CB 1348 and Thio-TEPA were compared, it was found that both drugs were equally effective, that is, no degree of difference was found to exist.

Mitotic suppression, pycnosis, and cell enlargement were observed in the normal tissue series sensitive to alkylating agents (TABLE 4). Cellular damage was evident in 1 of 3 culture series after treatment with CB 1348, in 1 of 4 with PAM, and in 3 of 4 with Thio-TEPA. Such gross cytocidal changes as total pycnosis, found in some neoplastic series, were never observed in the normal tissue series.

Discussion

The five agents under investigation produced cellular changes in HeLa cells that were qualitatively similar. The quantitative differences found to exist among these drugs were related to the effective millimolar concentrations required to cause significant changes in mitosis and giant cell formation. Results obtained in the 24-hour exposure series make it possible to ascribe a rank of cytotoxicity to each of the drugs, based on their efficacy per molecular weight. In decreasing order of effectiveness by weight they were listed as follows: HN2, TEM, Thio-TEPA, PAM, and CB 1348. This sequence correlates to some extent with the order of the LD_{50} values reported by Sternberg *et al.*[21] for

doses of TEM, HN2, Thio-TEPA, and CB 1348 given to adult rats. The concentrations of HN2, PAM, and Thio-TEPA found to be effective in tissue culture compare favorably with the doses of the same agents found to be effective in the treatment of cancer *in vivo* using the isolation and perfusion technique of drug administration.[22]

All agents evaluated produced a suppression of mitosis, aberrant chromosomal structures and giant cells. The similarity in changes caused by the group of drugs can be anticipated, since all agents possess chemical structures with two or more alkylating groups. As a group, the drugs may be considered to contain two components: (1) a carrier group, and (2) an alkylating moiety. The alkylating component was probably involved in the production of chromosomal aberrations, while the carrier group probably helped to modify the over-all cytotoxic properties of each agent.

Stimulation of mitosis was observed following exposure for 24 hours to very low concentrations of PAM and TEM. These observations are similar to the findings reported with TEM on chick fibroblasts.[23] Briefly, migration and mitosis were stimulated in chick cultures exposed for 24 hours to very low doses.

High concentrations of all drugs were cytocidal. Lower doses caused marked suppression of mitosis while minimal doses caused little or no change in mitosis. Lower mitotic values, when present, were usually the result of a decrease in normal metaphases, anaphases, and telophases, and an increase in aberrant metaphases. The most prominent mitotic alteration after 24 hours of drug exposure consisted of disorganization of chromosomes. Concomitantly, there was scatter or clumping to form eccentric basophilic groups within the cell. Chromosomes appeared "sticky" in that they adhered together in uneven, irregular groups. Anaphase bridges in the data reported for HeLa cells were apparently rare, as compared to chromosomal changes observed in chick fibroblasts treated with Thio-TEPA.[20] The chromosomal changes described were similar to those described by Koller[24] in dividing Walker carcinoma 256 cells treated with HN2 or with an aromatic nitrogen mustard. Koller reported that those cellular injuries that occurred not later than 48 hours after exposure to one single dose of drug, were true radiomimetic or nucleotoxic effects, for example, chromosome breaks and bridges. Those observed after 48 hours, for example, superfragmentation, were considered gross injuries and cytotoxic effects due to a metabolic disturbance induced in the cell by the drug. The effects of PAM on HeLa cells described in this report essentially are in agreement with the observations of Stubblefield and Hsu[25] on S-91 and Harding-Passey mouse melanomas and HeLa cells grown in tissue culture.

Cumulative cytological evidence indicates that successful completion of mitosis depends upon the smooth interaction of multiple cellular events. These include: (1) spindle formation and orientation, (2) relatively intact chromosomes attached to the spindle and orientated at precise time sequences, and (3) organized sol-gel movements to form a cleavage furrow. An aberration in one or more of these events could result in incomplete mitosis. The cytological alterations produced by the agents under study could account for the marked reduction in normally dividing HeLa cells.

The immediate cytological changes produced in HeLa cells and in primary human normal and neoplastic tissue culture cells by the alkylating agents, resembled those abnormalities produced by X rays[26,27,28] in embryonic chick sclerotic and choroid tissue cultures. The observed drug effects also generally agree with those effects of radiation by tritium (H^3) and yttrium (Y^{91}) on chick fibroblasts *in vitro*, as described by Stroud and Brues.[31] Both radiation and alkylating agents often caused chromosomal aberrations, mitotic suppression, "sticky" chromosomes, and pycnosis. While the consequences of treatment using these two types of agents were similar, no conclusions can be made from the data presented for a common mode of action.

Giant cells were formed in all HeLa cell cultures exposed to each of the five agents, and they resembled, in morphological detail, those giant cells formed in X-irradiated cultures of HeLa cells,[18,32] strain L mouse fibroblasts,[33] and the FL strain of human amnion cells.[34] Since normal mitoses were absent and aberrant mitoses were rare, it is probable that mitotic inhibition is an essential factor in the formation of giant cells in tissue cultures exposed to alkylating agents. Lea[35] states, for example, that an increase in the size of the cells, or of their nuclei, following irradiation has been observed in a number of materials, including bacteria and bean-root tips, and represents an inhibition of division without a change in growth rate. Loveless *et al.*[36] tested a large number of compounds, including antibiotics, antimetabolites, vitamins, and narcotics and found that only two, a nitrogen mustard and triethylene melamine, inhibited division without affecting growth in both yeast and *Escherichia coli*.

HeLa cells treated with drugs were maintained for 15 to 17 days after exposure as described in experiments by Puck and Marcus[18] with X-irradiated HeLa cells. During this period the drug-treated cells rapidly acidified the medium, indicating high acid production, characteristic of rapidly metabolizing cultures. The mechanism resulting in the formation of giant cells following drug exposure appears to be superficially similar to that involved in X- and gamma-radiation changes.[29,30] Puck and Marcus observed that the giant cells formed by X irradiation

had almost completely lost the ability to multiply, yet their biosynthetic equipment was still functioning, since such treated cells increased up to 15 times in volume. Till and Whitmore[33] exposed strain L fibroblasts grown in tissue culture to over 1,000 r and observed that the giant cells that formed were able to synthesize DNA, RNA, and protein. The increase in DNA content was accompanied by an increase in the mean chromosome number found for those cells attempting mitosis. They account for the failure of giant cells to divide on the basis of chromosomal aberrations and inhibited spindle formation. On the other hand, Bodenstein and Kondritzer,[37] using HN2, observed that the synthesis of DNA was suppressed or retarded while RNA synthesis was not affected in the enlarged cells formed in Amblystoma punctatum 8 days after drug exposure. HN2 caused mitotic suppression without effect on growth in Amblystoma punctatum *in vivo*, and in HeLa cells *in vitro*.

The cell enlargement observed in primary neoplastic and normal tissue cultures treated with a drug is in agreement with the data of Puck *et al.*,[38] who described giant cell formation in freshly isolated fibroblastic cells from normal tissues after X irradiation, and with the data of Plummer *et al.*,[23] who observed cell enlargement in first transfer chick fibroblast cultures after exposure to TEM.

A spectrum of cellular changes ranging from cytocidal injury to an absence of cellular changes were observed in human primary neoplastic and normal tissue cultures exposed to threshold concentrations of Thio-TEPA, CB 1348, and PAM. Thio-TEPA and CB 1348 were more effective damaging agents than PAM at the cellular level. These findings are in agreement with clinical data. Among the three alkylating agents currently being evaluated clinically, Thio-TEPA and CB 1348 appear to be more effective than PAM for the treatment of a wide variety of neoplasms.* In an investigation to determine the relationship between clinical and tissue culture results with chemotherapeutic agents, such as Thio-TEPA, on the course of human neoplastic disease, a significant degree of correlation appears to exist between the action of an agent on a tumor growing *in vivo* and on the same tumor growing *in vitro*.[39]

Summary

A comparative study has been made of the cellular changes produced in human normal and neoplastic cells in tissue culture by five alkylating agents currently useful in the chemotherapy of cancer. These agents are nitrogen mustard (HN2), triethylene melamine (TEM), triethylenethiophosphoramide (Thio-TEPA), chlorambucil (CB 1348) and phenylalanine nitrogen mustard (PAM).

*Wright, J. C. Personal Communication, 1959.

All agents produced changes in HeLa cells that were qualitatively similar and resembled changes described in cells exposed to X rays. Differences found to exist among the drugs were quantitative differences, related to effective millimolar concentrations required to cause significant damage. It was therefore possible to ascribe a rank of cytotoxicity to each drug, based on efficacy per molecular weight. In order of effectiveness by weight they were as follows: HN2, TEM, Thio-TEPA, PAM, and CB 1348.

Changes observed in HeLa cell cultures and in primary cultures of human, normal, and neoplastic tissues, consisted of suppression of mitosis, aberrant chromosomal structures, and formation of giant cells. An exposure time of one hour was sufficient to produce irreversible damage in all drug-exposed HeLa cells. High concentrations (1 mM) of all drugs were cytocidal to HeLa cells, while very low concentrations of TEM and PAM caused mitotic stimulation.

A comparison made by statistical methods of cellular injury caused by threshold concentrations of Thio-TEPA, CB 1348, and PAM in primary human normal and neoplastic cultures indicated that Thio-TEPA and CB 1348 were more effective damaging agents than PAM.

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